A taxonomic outline of the *Poecilimon affinis* complex (Orthoptera) using the geometric morphometric approach

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The genus *Poecilimon* contains 145 species, widely distributed in the Palaearctic, among which the *Poecilimon ornatus* group has the greatest diversity in the Balkans. Despite several revisions of the genus, the systematics of the species group, and in particular, of the taxa associated with the species *Poecilimon affinis*, is still unsolved. Due to morphological similarity, *P. affinis* with its subspecies, *P. nonveilleri* and *P. pseudornatus* form the *Poecilimon affinis* complex. The aim of this study is to test the hypotheses of an outlined species complex, namely the *P. affinis* complex, within the *P. ornatus* group using morphological data. Geometric analysis was conducted to explore variation in the structure of the tegmen, ovipositor, male cercus, and pronotum. A number of teeth and stridulatory file measurements provided additional information on morphological variation within the complex. A phylogenetic tree based on the cytochrome c oxidase subunit I gene (COI) was used for comparison with the morphological data. Canonical variate analysis showed that tegmen and male cercus are good morphostructures to distinguish the taxa belonging to the *P. affinis* complex from other species in the *P. ornatus* group. This may confirm our assumption for the designation of the *P. affinis* complex. The results of the principal component analysis of stridulatory file measurements, molecular data, and CVA of the ovipositor suggest adding two additional species to the complex: *P. ornatus* and *P. hoelzeli*.

1 **A taxonomic outline of the *Poecilimon affinis* complex**

2 **(Orthoptera) using the geometric morphometric**

3 **approach**

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22 **Abstract**

23 The genus *Poecilimon* contains 145 species, widely distributed in the Palaearctic, among which

24 the *Poecilimon ornatus* group has the greatest diversity in the Balkans. Despite several revisions

25 of the genus, the systematics of the species group, and in particular, of the taxa associated with

26 the species *Poecilimon affinis*, is still unsolved. Due to morphological similarity, *P. affinis* with

27 its subspecies, *P. nonveilleri* and *P. pseudornatus* form the *Poecilimon affinis* complex. The aim

28 of this study is to test the hypotheses of an outlined species complex, namely the *P. affinis*

29 complex, within the *P. ornatus* group using morphological data. Geometric analysis was

30 conducted to explore variation in the structure of the tegmen, ovipositor, male cercus, and

31 pronotum. A number of teeth and stridulatory file measurements provided additional information

32 on morphological variation within the complex. A phylogenetic tree based on the cytochrome c

33 oxidase subunit I gene (COI) was used for comparison with the morphological data. Canonical

34 variate analysis showed that tegmen and male cercus are good morphostructures to distinguish

35 the taxa belonging to the *P. affinis* complex from other species in the *P. ornatus* group. This may

36 confirm our assumption for the designation of the *P. affinis* complex. The results of the principal

37 component analysis of stridulatory file measurements, molecular data, and CVA of the ovipositor

38 suggest adding two additional species to the complex: *P. ornatus* and *P. hoelzeli*.

39

40 **Introduction**

41 *Poecilimon* Fischer, 1853 is one of the most species-rich genera within the Phaneropterinae

42 subfamily. This genus comprises 145 species distributed in the Palearctic region (*Cigliano et al.,*

43 *2021*). All species are short-winged and flightless herbivorous bush-crickets with complex

44 acoustic behavior (*Heller, 1990*). *Poecilimon* is currently divided into 18 species groups based

45 on molecular, morphological and bioacoustic data, while 16 species are not assigned to any of

46   them (*Cigliano et al., 2021*). The similarity and variability of morphological characteristics make

47   many *Poecilimon* species difficult to identify. *Poecilimon ornatus* group (13 species and 5

48   subspecies) (Fig. 1) is one of the groups for which the phylogenetic relationships between

49   species remain unclear and the status of several taxa is under discussion. Due to the reduced

50   wings and the influence of climatic and geomorphological factors, a rapid morphological

51   evolution took place in this group (*Chobanov & Heller, 2010*).

52   The first revision of *Poecilimon* was conducted by *Ramme (1933)*, who included taxa

53   from the currently recognized *Poecilimon ornatus* group in "Gruppe I." In *1984, Heller*

54   suggested dividing the group into eight taxa (*P. nobilis* Brunner von Wattenwyl, 1878; *P. obesus*

55   *obesus* Brunner von Wattenwyl, 1878; *P. obesus artedentatus* Heller, 1984; *P. affinis affinis*

56   (Frivaldszky, 1867); *P. affinis komareki* Cejchan, 1957; *P. affinis hoelzeli* Harz, 1966; *P. ornatus*

57   (Schmidt, 1850) and *P. pancici* Karaman, 1958; distributed mainly in the Balkans). Later, *P.*

58   *artedentatus* and *P. hoelzeli* were given species status (*Willemse, 1985; Willemse & Heller,*

59   *1992*), while *P. pancici* was synonymized (*Willemse, 1985*). Further, six new species were

60   described (*P. pindos* F. Willemse, 1982; *P. soulion* L. Willemse, 1987; *P. gracilioides* F.

61   Willemse & Heller, 1992; *P. jablanicensis* Chobanov & Heller, 2010; *P. pseudornatus* Ingrisch

62   & Pavicevic, 2010; *P. nonveilleri* Ingrisch & Pavicevic, 2010).

63   Among the *P. ornatus* group, *P. affinis* has the widest geographic range. It is distributed

64   from northern Greece to the Carpathians in Romania and an isolated spot in Ukraine. According

65   to *Cigliano et al. (2021)*, *P. affinis* consists of five subspecies (*P. affinis affinis* (Frivaldszky,

66   1868); *P. a. dinaricus* Ingrisch & Pavicevic, 2010; *P. a. hajlensis* Karaman, 1974; *P. a. komareki*

67   Cejchan, 1957; *P. a. serbicus* Karaman, 1974). *Karaman (1974)* reduced the status of *P. poecilus*

68   Ramme, 1951 to a subspecies of *P. affinis* and described two new subspecies: *P. a. serbicus* and

69   *P. a. hajlensis*. In *1984, Heller* suggested that *P. poecilus* and *P. a. affinis* are synonymous. Due

70   to doubts about the taxonomic status of *P. poecilus*, in the present study it will be treated

71   separately. *Poecilimon komareki* was described by Cejchan (1957), but *Heller (1984)* regarded it

72   as a subspecies of *P. affinis* because of their similarity. *Karaman (1972)* described *P. komareki*

73   *rumijae* based on the shape of the pronotum and body size. Because of the lowering of the status

74   of *P. komareki* to a subspecies of *P. affinis*, *P. k. rumijae* became synonymous of *P. a. komareki*,

75   which was confirmed by *Chobanov and Heller (2010)*. On the other hand, *Ingrisch & Pavicevic*

76   *(2010)* suggested regarding *P. rumijae* as a separate species, differing distinctly from *P. affinis*.

77   Morphological variability in these taxa was determined only based on minor differences in the

78   shape of the pronotum and body size (*Chobanov & Heller, 2010*). Furthermore, song of *P. a.*

79   *komareki* and *P. rumijae* resembles that of *P. pseudornatus* with a long silent beginning. Song of

80   *P. nonveilleri* is short with a typical structure, whereas *P. a. affinis* has also short song and shows

81   morphological differences to *P. nonveilleri* (own unpublished data). Due to the discrepancy

82   between the authors, *P. rumijae* will also be treated separately in the present study. *Poecilimon*

83   *pseudornatus*, *P. nonveilleri* and the subspecies of *P. affinis* are morphologically similar,

84   although a recent molecular study based on the cytochrome c oxidase I gene has shown that the

85   above taxa do not form a monophyletic group (*Kociński, 2020*). The lack of clear boundaries

86   between them and the unsolved phylogenetic relationship suggest that *P. pseudornatus*, *P.*

87   *nonveilleri* and subspecies of *P. affinis* should be treated as the *P. affinis* complex.

88   The 'species complex' is an informal taxonomic term showing the uncertainty of

89   taxonomic identification (*Sigovini et al., 2016*) and it is commonly used in insects (e.g., *Genier*

90   *& Moretto, 2017; Manani et al., 2017; Elfekih et al., 2018; Selnekovič & Kodada, 2019*). It may

91   be defined as a group of very closely-related taxa with similar morphology and difficult to

92   distinguish from one another. Taxa from a complex require a critical revision in order to clarify

93   the actual taxonomic position (*Sigovini et al., 2016*).

94   To determine the morphological variation of the *Poecilimon ornatus* group, especially

95   within the *Poecilimon affinis* complex, we used geometric morphometric methods based on the

96   shape variation of four structures: pronotum, male cercus, ovipositor, and tegmen (Fig. 2).

97   Geometric morphometrics is an approach that applies the landmark coordinates, which are the

98   correspondence points marked on a given morphostructure and are the same in all studied

99   specimens or species (*Bookstein, 1991; Dryden & Mardia, 1998*). This method considers the

100   spatial relationships between landmark variables, therefore providing more powerful statistical

101   results. It is also possible to find and analyze shape variations in the species within and between

102   populations (*Walker & Bell, 2000*). The geometric morphometric method has been proved to be

103   very useful for distinguishing species in insects (*Nunes et al., 2012; Prado-Silva et al., 2016; da*

104   *Silva et al., 2018*), especially in Orthoptera (*Romero, Rosetti & Remis, 2014; Barcebal et al.,*

105   *2015; Kaya, Boztepe & Ciplak, 2015; Kaya et al., 2015; Mugleston et al., 2016; Bian & Shi,*

106   *2018; Pan, Hong & Jiang, 2018; Liu, Chen & Liu, 2020*). The aim of the present study is to

107   assess the morphological diversity of the species within the *P. ornatus* group, outline morpho-

108   units and discuss the importance of morphological traits for the systematics of the group. We test

109   the hypothesis of the existence of the *P. affinis* complex.

110

Materials & Methods

112   **Specimen collection**

113   Bush-crickets were collected in the Balkan Peninsula (Bulgaria, Serbia, Montenegro, Albania,

114   North Macedonia, Greece) between 2017 and 2019 and stored in 96% ethanol (Table 1). In

115 Greece, field studies were approved by the Greek Ministry of the Environmental, Energy, and

116 Climate Change (No 154812/951).

117

118 **Geometric morphometrics**

119 In total, 196 specimens belonging to 16 taxa of the *Poecilimon ornatus* group were used for

120 geometric morphometric analyses. Four morphostructures (pronotum, male cercus, ovipositor,

121 and tegmen) were photographed using a stereomicroscope (Leica M165C) equipped with a

122 digital camera (Leica DMC5400) under strictly maintained magnification and resolution and

123 saved in jpg format. TPS files for each structure were created from the photographs with the

124 software tpsUtil v.1.26 following *Rohlf (2004)*. To explore the patterns of morphological

125 variation, 8 landmarks (including 1 semilandmark) of pronotum, 13 (7 semilandmarks) of cercus,

126 13 (1 semilandmark) of tegmen, and 9 (2 semilandmarks) of ovipositor (Fig. 2) were plotted

127 manually in tpsDIG2 v.2.17 (*Rohlf, 2015*). The list of landmarks and semilandmarks used in this

128 study is included in Table 2. After plotting the landmarks, the intersections marked in the TPS

129 files were aligned using a Procrustes superimposition. Partial warp scores were studied using

130 Canonical variate analysis (CVA) for each structure in MorphoJ v.1.06d (*Klingenberg, 2011*).

131 The first two Canonical Variables (CVs) with the greatest power to distinguish the groups were

132 plotted in the same software. The Mahalanobis distance was measured and statistically tested

133 using 10,000 permutation repeats.

134

135 **Stridulatory measurements**

136 The length of the stridulatory file and the number of stridulatory teeth were calculated for 154

137 specimens from the *P. ornatus* group (9 specimens of *P. affinis*, 24 – P. *affinis affinis*, 1 – *P.*

138   *affinis dinaricus*, 7 – *P. affinis hajlensis*, 5 – *P. affinis komareki*, 12 – *P. affinis serbicus*, 8 – *P.*

139   *hoelzeli*, 3 – *P. jablanicensis*, 15 – *P. nobilis*, 10 – *P. nonveilleri*, 12 – *P. obesus*, 10 – *P.*

140   *ornatus*, 29 – *P. pseudornatus*, 8 – *P. soulion*). Measurements were taken under

141   stereomicroscope with the aid of an ocular micrometer. For measurement of the stridulatory file

142   length, we used the distance from the first proximal (basal) to the last distal (apical) tooth. The

143   tegmen was placed upside down so that the stridulatory file could be viewed with its proximal

144   and distal ends being at the same level. This way, the distance between the ends was measured

145   along the imaginary line connecting those. The total number of stridulatory teeth and the number

146   of teeth within 2 mm at the middle of the stridulatory file were counted. Measurement data were

147   analyzed using Principal Component Analysis (PCA) in Past 4.03

148   (https://www.nhm.uio.no/english/research/infrastructure/past/).

149

150   **Phylogenetic analyses**

151   A fragment of the cytochrome c oxidase subunit I (COI) of mitochondrial DNA (mtDNA) was

152   used to determine the phylogenetic relationship between the taxa. We aimed to construct a

153   phylogenetic tree focusing on the species of the *P. affinis* complex. A total of 71 sequences of 14

154   *Poecilimon* taxa were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The

155   DNA sequences were aligned using CodonCode Aligner 9.0.2

156   (https://www.codoncode.com/aligner) with default parameters. The maximum likelihood (ML)

157   and Bayesian inference (BI) analyses were used to infer the phylogenetic relationships. The best-

158   fit model of nucleotide substitution was determined with jModelTest2 (*Guindon & Gascuel,*

159   *2003; Darriba et al., 2013*). ML was performed in IQ-TREE (*Nguyen et al., 2015*), whereas BI

160   in MrBayes 3.2. (*Ronquist et al., 2012*). For bootstrap analyses, 1,000 pseudoreplicates were

161 generated. BI was carried out with 10,000,000 generations, with a sampling of trees every 100

162 generations. Likelihood values were observed with Tracer v.1.7 (*Rambaut et al., 2018*). The tree

163 was visualized in FigTree 1.4.4 (*Rambaut, 2018*).

164

165 **Results**

166 **Morphology**

167 As a result, 54 images of ovipositor, 130 of tegmen, 142 of pronotum, and 141 of male cercus

168 were used in the analyses. In some specimens, tegmen and male cercus were damaged and not

169 used for this study. The landmarks were chosen based on the shape and structure of the

170 ovipositor (7 landmarks, 2 semilandmarks) (Fig. 2A), male cercus (6 landmarks, 7

171 semilandmarks) (Fig. 2B), pronotum (7 landmarks, 1 semilandmark) (Fig. 2C), and tegmen (12

172 landmarks, 1 semilandmark) (Fig. 2D).

173   CV analysis of the tegmen (Fig. 3) revealed significant variation within the *P. ornatus* group

174 and *P. affinis* complex. At the species group level, the first two CV analyses together accounted

175 for 77.72% of the total variation (CV1=55.64%, CV2=22.08%). A combination of the results of

176 the CV1 and CV2 analyses of the tegmen separated the species *P. hoelzeli*, *P. obesus*, *P.*

177 *jablanicensis* and *P. nobilis* from the other species of the *Poecilimon ornatus* group and revealed

178 an overlap between *P. pseudornatus*, *P. poecilus*, *P. nonveilleri*, and *P. affinis* (Fig. 3A). The

179 Mahalanobis distance obtained through pairwise comparisons among the group revealed highly

180 significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.50 (*P. affinis* and

181 *P. pseudornatus*) to 19.66 (*P. poecilus* and *P. obesus*). The Procrustes distances also showed

182 significant differences between groups (10,000 permutation rounds; P<0.0001) ranging from

183 0.03 (*P. poecilus* and *P. pseudornatus*) to 0.28 (*P. nobilis* and *P. obesus*) (Table S1).

184   At the species complex level, the first two CVs together accounted for 47.9% of the total

185   variation of the tegmen (CV1=28.5% and CV2=19.4%). CV1 and CV2 analyses of the

186   *Poecilimon affinis* complex did not indicate clear clusters representing each of the existing

187   species/subspecies. However, the specimens of *P. a. affinis* show differentiation in terms of their

188   occurrence (Bratiya, Kirilova Polyana, Yavorow-Pirin, Osogovo, Rila) in contrast to *P.*

189   *pseudornatus*, where specimens from different localities (Kamena Gora, Durmitor, Treschnievik,

190   Vusanje) are grouped together (Fig. 3B). The Mahalanobis distances between taxa for tegmen

191   are 2.77 for *P. poecilus* and *P. pseudornatus*, and 8.13 for *P. a. komareki* and *P. a. dinaricus*

192   (10,000 permutation rounds; P<0.0001). The Procrustes distances also showed significant

193   differences (10,000 permutation rounds; P<0.001), ranging from 0.03 (*P. a. serbicus* and *P.*

194   *pseudornatus*) to 0.12 (*P. rumijae* and *P. a. dinaricus*) (Table S2).

195   For the ovipositor, at the species group level, the first two CVs together accounted for

196   78.43% of the total variation (CV1=54.78%, CV2=23.65%) (Fig. 4A). The scatter plot from CV1

197   and CV2 shows that species from the *Poecilimon affinis* complex cannot be clearly separated

198   from other species of the *Poecilimon ornatus* group (Fig. 4A). The Mahalanobis distances

199   obtained by pairwise comparisons among group revealed highly significant differences (10,000

200   permutation rounds, P<0.0001), ranging from 2.78 (*P. poecilus* and *P. hoelzeli*) to 15.72 (*P.*

201   *gracilis* and *P. nobilis*). The Procrustes distances also showed significant differences between

202   groups (10,000 permutation rounds, P<0.0001) ranging from 0.04 (*P. affinis* and *P. hoelzeli*) to

203   0.19 (*P. pseudornatus* and *P. gracilis*) (Table S3).

204   At the species complex level, the first two CVs together accounted for 83.92% of the total

205   variation of the ovipositor (CV1=70.26% and CV2=13.66%) (Fig. 4B). The centroid size (the

206   square root of the sum of the squared distances of all landmarks from their centroid) of CV1 and

207 CV2 shows that species from the *Poecilimon affinis* complex can be clearly separated from each

208 other (Fig. 4B). The Mahalanobis distances obtained through pairwise comparisons of the

209 complex revealed highly significant differences (10,000 permutation rounds; P<0.0001), ranging

210 from 2.69 (*P. rumijae* and *P. a. affinis*) to 14.50 (*P. pseudornatus* and *P. a. hajlensis*). The

211 Procrustes distances also showed highly significant differences (10,000 permutation rounds;

212 P<0,005), ranging from 0.03 (*P. a. serbicus* and *P. a. affinis*) to 0.15 (*P. a. komareki* and *P. a.*

213 *dinaricus*) (Table S4).

214   CV analysis of the male cercus (Fig. 5) also revealed significant variation within the *P.*

215 *ornatus* group and the *P. affinis* complex. At the group level, the first two CVs together

216 accounted for 69.82% of the total variation (CV1=40.59%, CV2=29.23%). The scatter plot from

217 CV1 and CV2 shows that species from the *Poecilimon affinis* complex can be clearly separated

218 from other species of the *Poecilimon ornatus* group **(**Fig. 5A). The Mahalanobis distances

219 obtained through pairwise comparisons among group revealed highly significant differences

220 (10,000 permutation rounds; P<0.0001), ranging from 2.71 (*P. pseudornatus* and *P. affinis*) to

221 12.25 (*P. hoelzeli* and *P. jablanicensis*). The Procrustes distances also showed significant

222 differences between groups (10,000 permutation rounds; P<0.0001), ranging from 0.03 (*P.*

223 *affinis* and *P. pseudornatus*) to 0.17 (*P. pseudornatus* and *P. nobilis*) (Table S5).

224   For the male cercus, at the complex level, the first two CVs together accounted for 54.33% of

225 the total variation (CV1=30.38% and CV2 =23.95%). The centroid size of CV1 and CV2 shows

226 that only *P. a. affinis*, *P. rumijae*, *P. a. komareki*, and *P. nonveilleri* can be clearly separated

227 from other members of the *P. affinis* complex (Fig. 5B). The Mahalanobis distances obtained

228 through pairwise comparisons of the complex revealed significant differences (10,000

229 permutation rounds; P<0.0001), ranging from 2.87 (*P. pseudornatus* and *P. a. hajlensis*) to 8.65

230   (*P. a. dinaricus* and *P. a. komareki*). The Procrustes distances also showed significant

231   differences (10,000 permutation rounds; P<0.0001), ranging from 0.03 (*P. a. affinis* and *P.*

232   *poecilus*) to 0.10 (*P. a. komareki* and *P. nonveilleri*) (Table S6).

233   For the pronotum, at the group level, the first two CVs together accounted for 75.84% of the

234   total variation (CV1 =57.24%, CV2=18,60%) (Fig. 6). The scatter plot from CV1 and CV2

235   shows that species from the *Poecilimon affinis* complex cannot be clearly separated from other

236   species of the *Poecilimon ornatus* group (Fig. 6A). The Mahalanobis distances obtained through

237   pairwise comparisons among group revealed significant differences (10,000 permutation rounds;

238   P<0.0001), ranging from 2.20 (*P. poecilus* and *P. affinis*) to 12.81 (*P. gracilis* and *P. obesus*).

239   The Procrustes distances also showed significant differences between groups (10,000

240   permutation rounds; P<0.0001), ranging from 0.03 (*P. poecilus* and *P. affinis*) to 0.16 (*P. gracilis*

241   and *P. jablanicensis*) (Table S7).

242   At the complex level, the first two CVs together accounted for 72.01% of the total

243   variation of the pronotum (CV1=46.56% and CV2 =25.45%). The centroid size of CV1 and CV2

244   shows that only *P. rumijae* can be clearly separated from other species from the *P. affinis*

245   complex (Fig. 6B). The Mahalanobis distances obtained through pairwise comparisons of the

246   complex revealed significant differences (10,000 permutation rounds; P<0.0001), ranging from

247   2.73 (*P. a. hajlensis* and *P. a. affinis*) to 5.68 (*P. rumijae* and *P. nonveilleri*). The Procrustes

248   distances also showed highly significant differences (10,000 permutation rounds; P<0.0001),

249   ranging from 0.04 (*P. poecilus* and *P. a. affinis*) to 0.14 (*P. rumijae* and *P. nonveilleri*) (Table

250   S8).

251

252   **Stridulatory measurements**

253   *Poecilimon soulion* and *P. jablanicensis* have the shortest stridulatory file of all studied species

254   (2.74 – 3.17 and 2.96 – 3.04, respectively). In contrast, *P. affinis komareki* has the longest

255   stridulatory file (5.34 – 5.88) and the greatest number of teeth on its structure (158 –195).

256   *Poecilimon obesus* has the lowest number of teeth, which proves that the length of the

257   stridulatory file does not correlate with the number of teeth (Table 3). Principal Component

258   Analysis of the stridulatory file and the number of teeth shows that *P. nonveilleri, P. ornatus, P.*

259   *hoelzeli, P. pseudornatus, P. a. serbicus, P. a. hajlensis*, and *P. a. affinis* overlap. Moreover, we

260   can conclude that *P. a. affinis* is the most diverse taxon within the *P. ornatus* group, while *P. a.*

261   *komareki* is the most distinct taxon of the studied group (Fig. 7).

262

263   **Phylogenetic analyses**

264   The final alignment consists of 607 bp, of which 450 were conservative, 157 variable and 83

265   parsimony-informative sites. HKY+G was selected as the best-fit evolution model for site

266   substitution. The topologies obtained from BI and ML analyses were similar. Bootstrap values

267   (ML) (>50%) and BI posterior probabilities (>0.5) are shown on the nodes of the tree presented

268   on Fig. 8. To root the tree, *Poecilimon cervus* Karabag, 1950, belonging to the *Poecilimon*

269   *bosphoricus* Brunner von Wattenwyl, 1878 species group, was chosen. The BI and ML trees

270   based on the COI data show that the *P. affinis* complex forms a paraphyletic group. The most

271   diverse taxon in the complex is *P. a. affinis*, occupying different nodes on the phylogenetic tree

272   due to its place of occurrence. *Poecilimon a. affinis* from Kirilova Polyana (Bulgaria, Rila Mtns)

273   occupies a basal position in the tree and seems to be a sister taxon to the remaining taxa of the

274   complex. Two species of the *P. ornatus* group, preliminary left outside the *P. affinis* complex, *P.*

275   *ornatus* and *P. hoelzeli,* were placed within the same clade (Fig. 8).

276

Discussion

278 **Morphology**

279 This work aimed to determine the morphological characteristics that separate bush-crickets

280 belonging to the *P. affinis* complex from other species of the *P. ornatus* group through the

281 geometric morphometrics approach. The morphology of the tegmen, ovipositor, male cercus and

282 pronotum were used successfully in morphological studies of *Poecilimon* (*Heller, 2004*;

283 *Chobanov & Heller, 2010*; *Kaya et al., 2012*; *Kaya, Boztepe & Ciplak, 2015*; *Kaya et al., 2018*).

284 The present work showed that the studied morphostructures can partly be used to separate taxa of

285 the species rank in the *Poecilimon ornatus* group. *Chobanov & Heller (2010)* noticed that the

286 pronotal shape and the size of the area of the tegmen covered by the pronotum vary between

287 specimens from the same locality. Our results support the poor taxonomic utility of the shape of

288 pronotum in this group for distinguishing the species belonging to the *P. affinis* complex from

289 other species in the group (Fig. 6A). However, based on the shape of the tegmen, *P. affinis* and

290 its subspecies group with *P. nonveilleri*, *P. pseudornatus* in the same place, which clearly

291 separates them from other species (Fig. 3A). This may confirm our assumption for the

292 designation of the *P. affinis* complex including other species from the *Poecilimon ornatus* group.

293 CV analysis of centroid sizes of the pronotum (Fig. 6B) shows that *P. rumijae* is the most

294 distinct taxon among the *P. affinis* complex, and does not overlap with *P. a. komareki*.

295 *Poecilimon rumijae* may likely be treated as a separate species of the *P. ornatus* group, differing

296 distinctly from subspecies of *P. affinis (Ingrisch & Pavicevic, 2010)*, but further studies are

297 required to confirm its taxonomic position. This assumption is also confirmed by the analysis of

298 the ovipositor, where *P. a. komareki* is more similar to *P. a. dinaricus* and *P. pseudornatus*,

299   whereas *P. rumijae* is more similar to *P. a. affinis* (Fig. 4B). On the other hand, the results based

300   on the male cercus (Fig. 5B) show that *P. a. komareki* and *P. rumijae* overlap, which proves high

301   similarities within this morphostructure and may confirm the accuracy of lowering *P. rumijae* to

302   the rank synonymous with *P. a. komareki* (*Chobanov & Heller, 2010*; *Cigliano et al., 2021*).

303   *Ingrisch & Pavicevic (2010)* considered *P. rumijae* to be similar to *P. nonveilleri* and *P. affinis*.

304   Our results confirm a close relationship between *P. rumijae* and *P. affinis*, but not between *P.*

305   *rumijae* and *P. nonveilleri*, which, according to all morphostructures, are the most distant from

306   each other (Fig. 3A, 4A, 5A, 6A).

307   The most distinct species in our sample is *P. nobilis* based on the analysis of the tegmen

308   (Fig. 3A) and male cercus (Fig. 5A), *P. gracilis* based on the ovipositor (Fig. 4A), and *P. obesus*

309   based on the pronotum (Fig. 6A), which suggest not to include these species in the *P. affinis*

310   complex. On the other hand, *P. affinis* is the most diffuse taxon in the group (Fig. 3A, 4A, 5A,

311   6A). The results suggest that the difference between specimens of *P. a. affinis* is related to the

312   locality in which they occur (Fig. 3B), and is generally connected with altitude (*Chobanov &*

313   *Heller, 2010*). Specimens of *P. a. affinis* from Pirin are distant from individuals from Bratiya,

314   Kirilowa Polyana, Osogovo, Rila and are more closely related to *P. poecilus*, *P. a. hajlensis* and

315   *P. a. komareki* (Fig. 3B). On the other hand, the position of the centroid size of *P. pseudornatus*

316   from different localities (Durmitor, Kamena Gora, Treshnievik, Vusanje) overlaps, which proves

317   a lower morphological variability in terms of location than in the case of *P. a. affinis* (Fig. 3B).

318   At the group level based on the male cercus (Fig. 5A), species from the *P. affinis* complex (*P.*

319   *affinis* with its subspecies, *P. nonveilleri* and *P. pseudornatus*) overlap. Thus, this is the second

320   morphostructure to confirm the existence of this complex. Additionally, *Chobanov & Heller*

321   *(2010)* suggested that the male cercus may be a better feature for separating species in this group.

322   The results of the CV analysis of centroid size of the ovipositor (Fig. 4A) show the similarity

323   between *P. affinis*, *P. hoelzeli*, *P. pseudornatus*, *P. poecilus*, and *P. nonveilleri*, which may

324   indicate the extension of the *P. affinis* complex with *P. hoelzeli* (Fig. 4A). *Poecilimon poecilus*,

325   which we suggested to treat separately in this work, seems to fell within the variation of *P. a.*

326   *affinis*. It is confirmed by all the morphostructures studied, where *P. poecilus* overlaps with other

327   subspecies: *P. a. affinis, P. a. hajlensis, P. a. komareki* (Fig. 3A, 4A, 5A, 6A). However, to

328   establish the taxonomic status of *P. poecilus*, additional research is needed.

329

330   **Stridulatory structures measurements**

331   The stridulatory file and the number of teeth can be a good morphological feature for

332   distinguishing taxa in the *P. ornatus* group (*Heller, 1984; Willemse, 1985; Heller, 1988;*

333   *Chobanov & Heller, 2010*). *Heller (1988)* reports that *P. ornatus* has fewer teeth than *P. affinis,*

334   about 158-212, with some exceptions of large specimens having up to 220 teeth, as confirmed by

335   our results (Table 3). The length of stridulatory file is the same in both species and averaged

336   4.08. Thus, this morphostructure and the number of teeth are not a good feature for

337   distinguishing *P. affinis* from *P. ornatus*. *Heller (1984)* observed about 220-230 teeth in *P.*

338   *affinis* species, while *Chobanov & Heller (2010)* observed 180-240. They suggest that the

339   number is generally more variable in southeastern populations (SW Bulgaria). The lowest

340   number of teeth is found in small specimens from high altitudes. Principal Component Analysis

341   (PCA) shows a similarity between three subspecies (*P. a. affinis, P. a. serbicus* and *P. a.*

342   *hajlensis*) (Fig. 7). On the other hand, *P. a. komareki* does not overlap with other subspecies,

343   which may mean that it is the most distinct taxon from all studied taxa of the *P. ornatus* group.

344   *Poecilimon hoelzeli* and *P. pseudornatus* have a similar number of teeth and length of the

345   stridulatory file. *Poecilimon ornatus, P. nonveilleri, P. a. affinis, P. a. hajlensis, P. a. serbicus,*

346   *P. pseudornatus* and *P. hoelzeli* overlap, which can suggest that *P. hoelzeli* and *P. ornatus* should

347   be included in the designated *P. affinis* complex.

348

349   **Phylogenetic data**

350   The first genetic studies using ribosomal internal transcribed spacers (ITS1 and 2)

351   involving some of the group's species were conducted by *Ullrich et al. (2010)*. However, they

352   did not provide conclusive information on the relationship between species in this group.

353   *Kocinski (2020)* performed a genetic analysis based on the cytochrome c oxidase I gene (COI) of

354   the *P. ornatus* group, and confirmed the monophyly of this group. Our results, focusing on

355   species from the *P. affinis* complex, show that it forms a paraphyletic group (Fig. 8). Two

356   additional species, *P. hoelzeli* and *P. ornatus*, are distributed with the other taxa of the complex,

357   thus they probably should be included in the *P. affinis* complex determined previously. This

358   assumption is similar to the results of the CVA of the ovipositor, where taxa from the complex

359   overlap with *P. hoelzeli* (Fig. 4A). Moreover, based on the phylogenetic tree (Fig. 8), *P. a. affinis*

360   is the most diverse species in the complex, occupying different nodes, which is supported by the

361   CVA results of the tegmen (Fig. 3B). The variability is related to the location (Bratiya, Kirilova

362   Polyana, Rila, Yavorow) of the populations of *P. a. affinis*, and is connected with the altitude of

363   occurrence (*Chobanov & Heller, 2010*). *Poecilimon a. komareki* and *P. rumijae* form different

364   nodes, which may suggest treating them as separate taxa of the *P. ornatus* group. This opinion is

365   confirmed by the CVA results of pronotum and ovipositor (Fig. 4B, 6B). The specimens from *P.*

366   *poecilus* also form different nodes compared to *P. a. affinis*, thus, it may be treated as a

367 subspecies of *P. affinis*, which is supported by the CVA of the tegmen, male cercus, ovipositor,

368 and pronotum (Fig. 3B, 4B, 5B, 6B).

Conclusions

370 The geometric morphometric method has proven to be useful in studying the morphological

371 diversity of bush-crickets. Combined with the analysis of the stridulatory file and molecular

372 phylogeny, it provides better insight into the relationship between species from the *Poecilimon*

373 *ornatus* group, and in particular, the taxa of the *Poecilimon affinis* complex. Morphological

374 analysis of selected morphostructures and molecular data showed the paraphyly of the *P. affinis*

375 complex unless *P. ornatus* and *P. hoelzeli* are included. Additionally, the taxonomic status of the

376 *P. rumijae* and *P. poecilus* species remains unclear. Our results show some discordances with

377 previous studies and point to the need for a most thorough interdisciplinary phenetic and genetic

378 study in order to solve the systematics of this particular group of bush-crickets.

379

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384

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505

Figure 1

Representatives of the studied taxa from the *Poecilimon ornatus* group.

(A) *P. affinis hajlensis.* (B) *P. affinis affinis.* (C) *P. hoelzeli.* (D) *P. rumijae.* (E) *P. nonveilleri.* (F) *P. poecilus.* (G) *P. pseudornatus.* (H) *P. ornatus*. Photos D. Chobanov.

Figure 2

Position of the landmarks (red dots) on *Poecilimon* species used for geometric morphometrics.

(A) ovipositor. (B) male cercus. (C) pronotum. (D) tegmen.
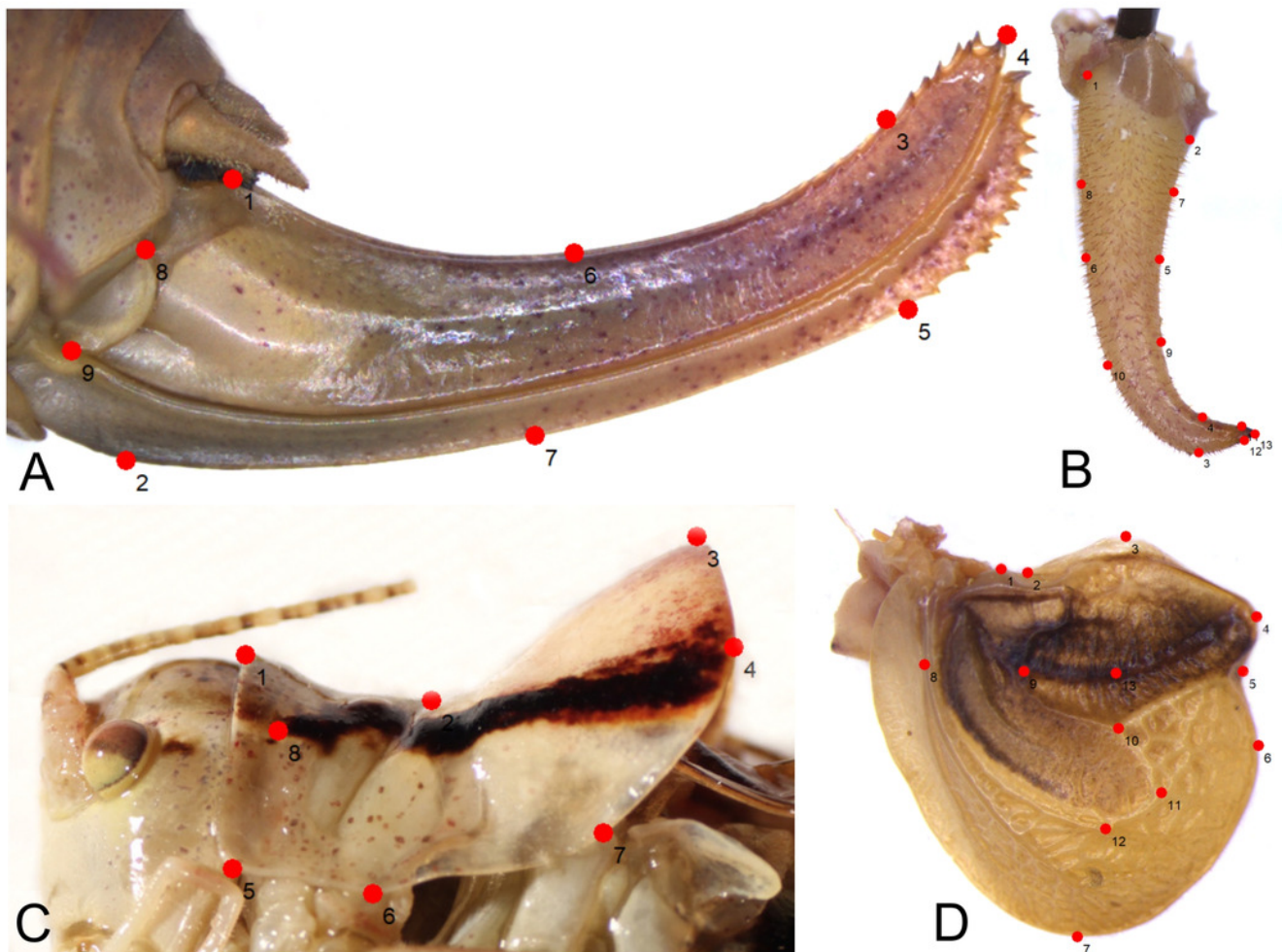
Figure 3

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of tegmen:*P. ornatus* group (A) and *P. affinis* complex (B).

The different colors of the species *P. pseudornatus* and *P. a. affinis* indicate different locations from which the specimens were collected. The localities are indicated below taxa name (SR – Serbia, MN – Montenegro, BG – Bulgaria).

off
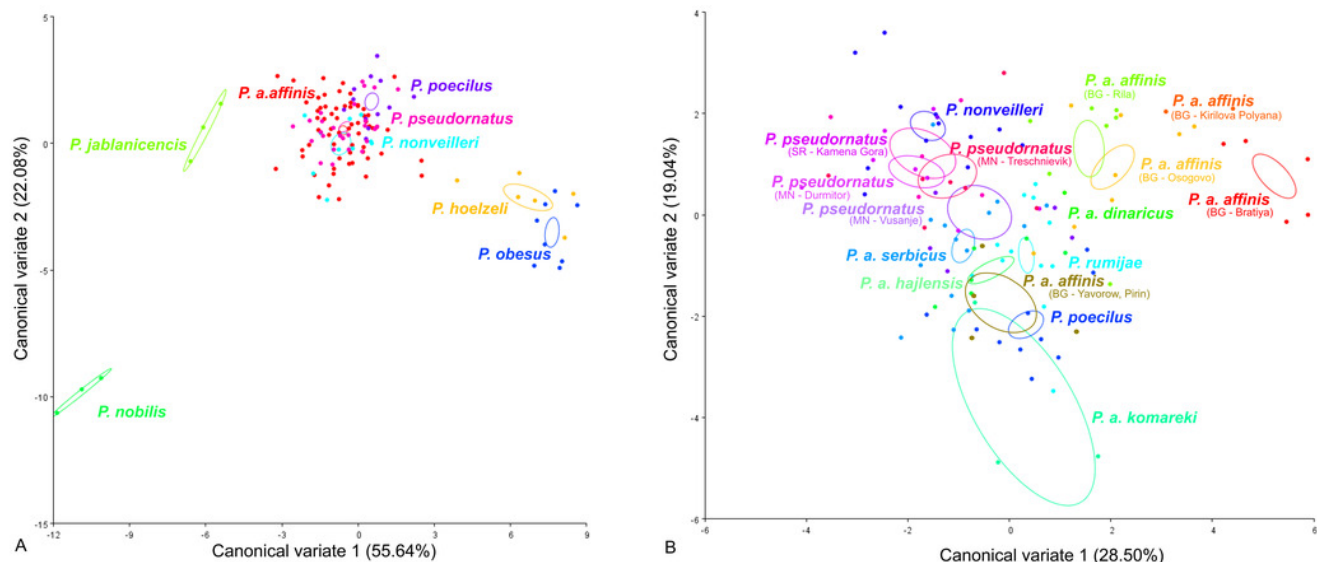
Figure 4

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of ovipositor: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of studied bush-crickets.
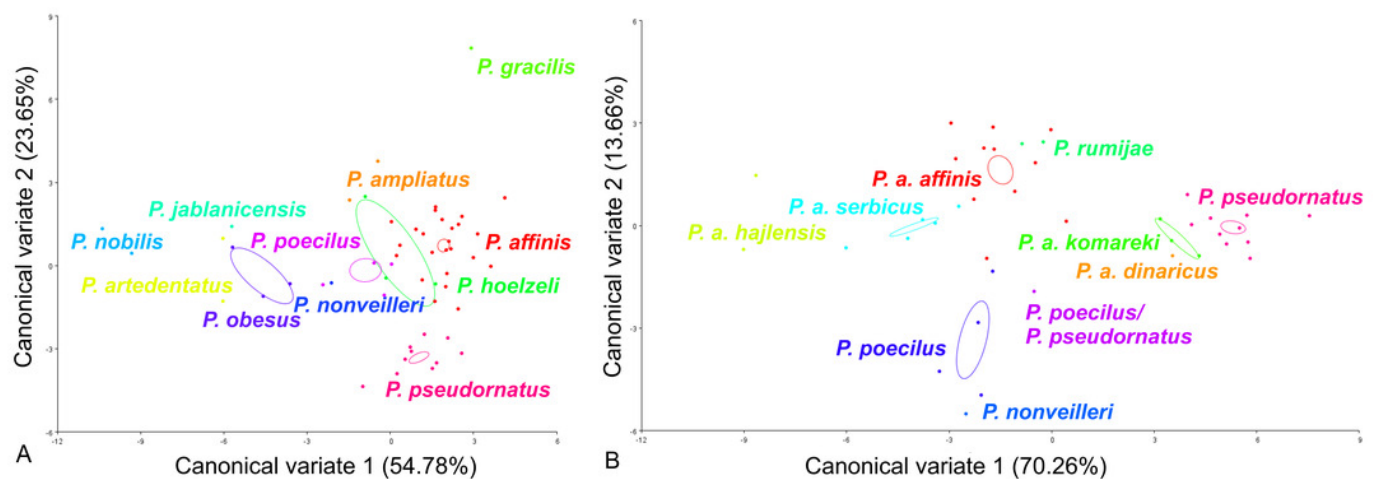
Figure 5

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of male cercus: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of the studied bush-crickets.
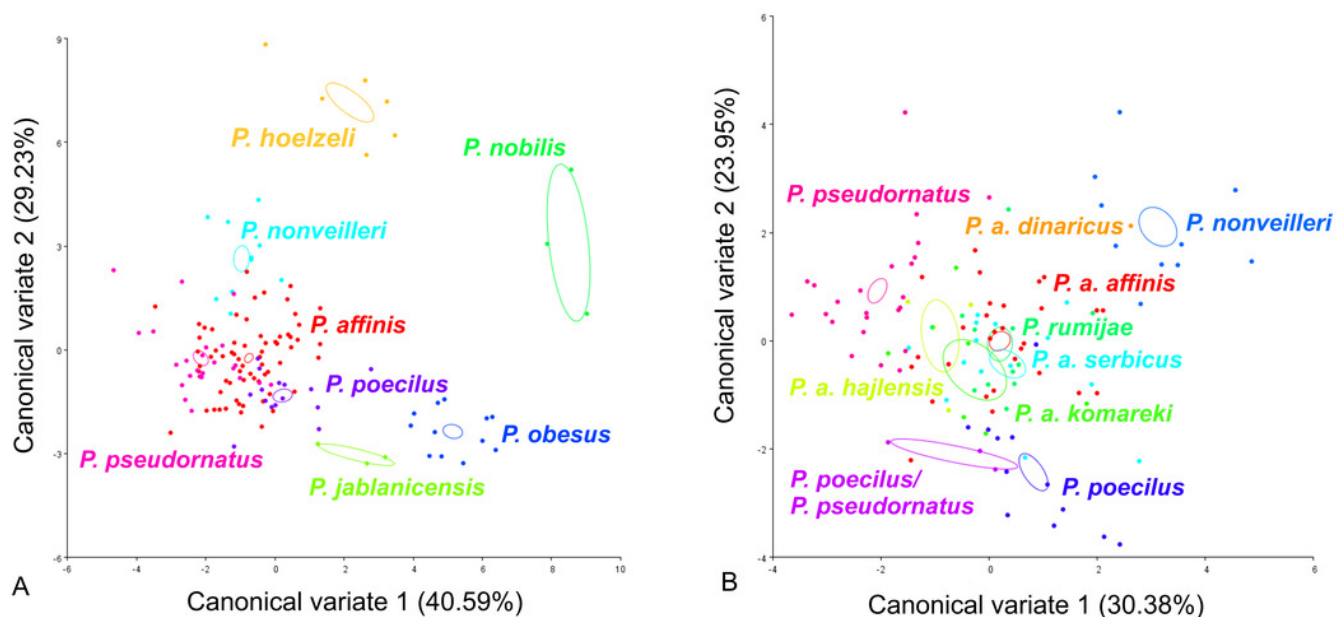
Figure 6

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of pronotum: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of the studied bush-crickets.
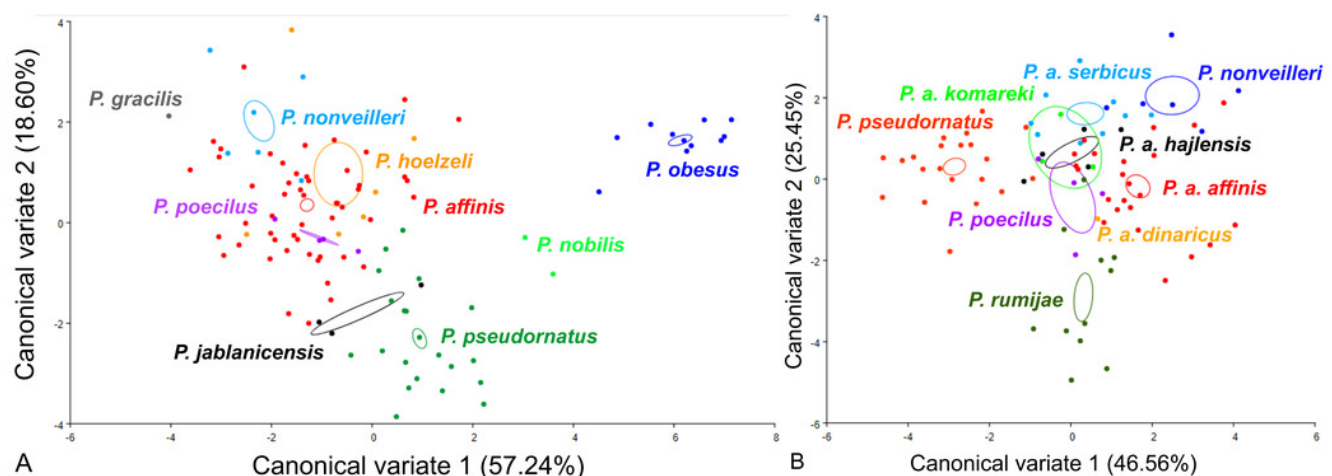
Figure 7

Principal Component Analysis (PCA) of stridulatory measurements and number of teeth: *P. ornatus* group.

The different colors indicate different species/subspecies of studied bush-crickets.
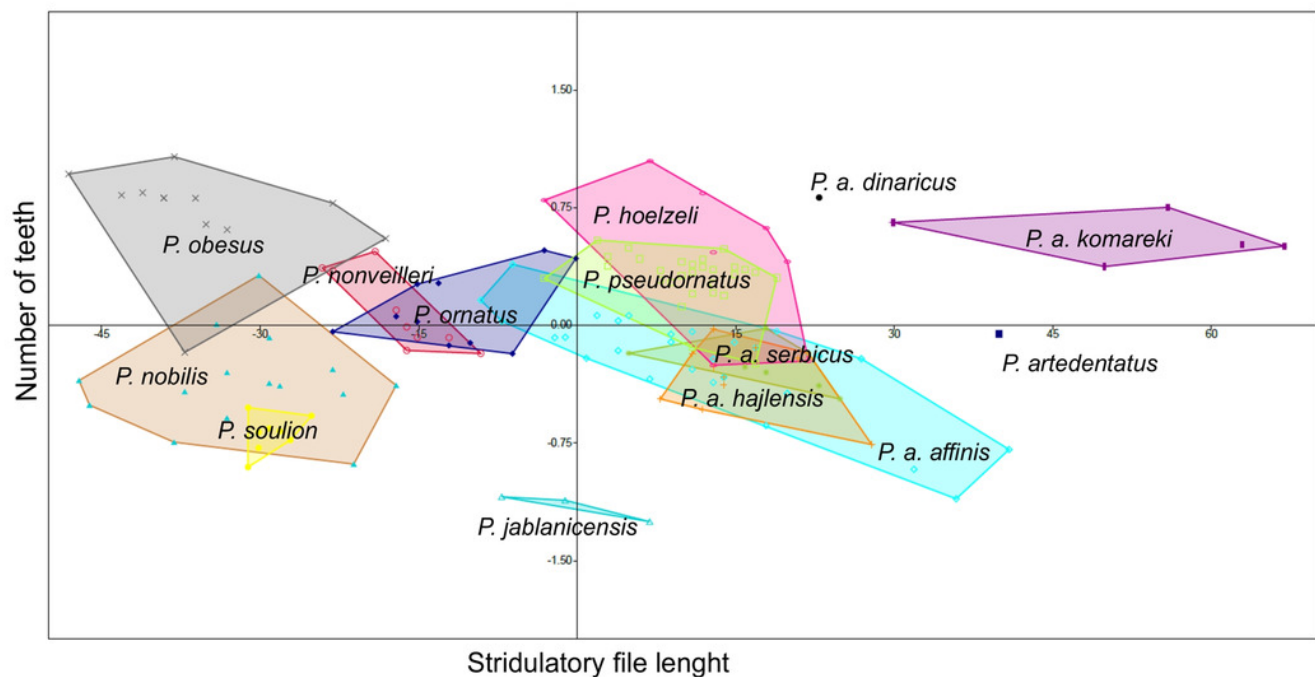
Figure 8

Phylogenetic tree based on Bayesian inference method including COI sequences of the *P. affinis* complex.

Bayesian Inference (BI) and Maximum Likelihood (ML) topologies were similar, so only one tree is shown. BI posterior probability (>0.5) and ML bootstrap values (>50) are shown on the nodes. Pink indicates species outside of the *P. affinis* complex; blue indicates taxa from the designated *P. affinis* complex.

Phylogenetic tree with tip labels and clade annotations:

P. a. komareki — Paffkomluc1, Paffkomluc2, Paffkomluc3 (1/100, 0.9/82)
P. a. dinaricus — Paffdinsus1
P. nonveilleri — Pnonveisus1 (0.99/87)
P. poecilus — Paffpoepop1, Paffserlju1 (1/98)
P. a. serbicus — Paffpoepop2, Paffpoepop3 (0.95/98)
Ppseudovus1, Ppseudovus2 (1/100)
Ppseudovus3
Ppseudodur1, Ppseudodur3 (1/92)
Ppseudodur2
Ppseudokam1
P. pseudornatus — Ppseudotre2
Ppseudotre3
Ppseudotre1 (0.57/75)
Ppseudokam3
P. a. hajlensis — Paffhajhaj1, Paffhajhaj2, Paffhajhaj3
Ppseudohaj1, Ppseudohaj2, Ppseudohaj3
Ppseudokam2, Ppseudokam4, Ppseudokam5
P. pseudornatus
P. rumijae — Paffrumkol1, Paffrumkol2, Paffrumkol3 (0.78/64)
P. a. serbicus — Paffserlju2, Paffserlju3
P. nonveilleri — Pnonveisus2, Pnonveisus3 (0.66/73)
P. a. affinis — Paffaffyav1, Paffaffyav2, Paffaffyav3
P. a. affinis — Paffaffril1, Paffaffril2, Paffaffril3 (1/100, 0.62/63)
P. a. affinis — Paffaffoso1, Paffaffoso2, Paffaffoso3 (0.92/98, 1/98, 0.67/64)
P. a. affinis — Paffaffbra1, Paffaffbra2, Paffaffbra3 (1/99, 1/98)
P. ornatus — Pornatuche2, Pornatuche3 (1/98)
P. a. dinaricus — Paffdinmra1 (0.8/98, 0.99/96)
P. hoelzeli — Phoelzegen1 (1/98)
P. a. affinis — Paffaffkir1, Paffaffkir2 (1/97, 0.82/76)
P. gracilioides — Pgracilioi1 (1/100)
P. obesus — Pobesusgen1
P. cervus — Pcervusgen1

The number of specimens used for the geometric morphometric analysis.

1 Table 1:

2 The number of specimens used for the geometric morphometric analysis.

Species	Male cercus	Tegmen	Ovipositor	Pronotum
*Poecilimon affinis affinis** (Frivaldszky, 1868)	29	26	11	23
*Poecilimon affinis komareki** Cejchan, 1957	6	3	3	3
*Poecilimon affinis dinaricus** Ingrisch & Pavićević, 2010	1	1	1	1
*Poecilimon affinis serbicus** Karaman, 1974	14	14	5	9
*Poecilimon affinis hajlensis** Karaman, 1974	4	6	2	5
*Poecilimon affinis poecilus** Ramme, 1951	15	12	5	4
*Poecilimon rumijae** Karaman, 1972	12	12	2	11
*Poecilimon nonveilleri** Ingrisch & Pavićević, 2010	10	10	1	6
*Poecilimon pseudornatus** Ingrisch & Pavićević, 2010	24	26	10	21
Poecilimon hoelzeli Harz, 1966	6	6	3	6
Poecilimon jablanicensis Chobanov & Heller, 2010	3	3	1	3
Poecilimon nobilis Brunner von Wattenwyl, 1878	3	3	2	2
Poecilimon obesus Brunner von Wattenwyl, 1878	12	8	3	11
Poecilimon gracilis (Fieber, 1853)	-	-	1	1
Poecilimon artedentatus Heller, 1984	-	-	2	-

4 *- *Poecilimon affinis* complex

List of the landmarks and semilandmarks of the pronotum, male cercus, tegmen, and ovipositor used in the geometric morphometric analysis.

1 Table 2:

2 List of the landmarks and semilandmarks of the pronotum, male cercus, tegmen, and ovipositor
3 used in the geometric morphometric analysis.

The landmark number	Pronotum	Male cercus	Tegmen	Ovipositor
1	upper frontal part	groove left at base	most distant point	highest point at the base
2	upper part of mid groove	groove right at base	upper concave point	lowest point of the base
3	upper posterior point	most distant point at apex	most distant point	begging of teeth at the upper valve
4	lateral posterior point	opposite to 3*	most distant point	tip of upper valve
5	lower frontal part	middle measured approximately between 4 and 2*	concave side point	begging of teeth at the lower valve
6	lowest middle part	opposite to 5*	most distant point	middle between 1 and 3*
7	mid point between 4 and 6*	approximetly middle between 2 and 5*	most distant point	middle between 2 and 5*
8	begging of dark band	approximetly middle between 1 and 6*	most distant point of the lateral vein	upper point of gonangulum
9		approximetly middle between 5 and 4*	bifurcation between veins	lower point of gonangulum
10		approximetly middle between 6 and 3*	bifurcation between veins	
11		upper end of black spine	bifurcation between veins	
12		lower end of black spine	bifurcation between veins	
13		tip of cercus	mark on the stridulatory vein between the points 3 and 10*	

4   *- semilandmarks

Measurements for stridulatory files of *P. ornatus* group. Measurements are given in mm: first row – Min-Max values. in brackets – avarage ± Standard deviation.

Measurements are given in mm: first row – Min-Max values. in brackets – avarage ± Standard deviation.

1 Table 3:
2 Measurements for stridulatory files of the *P. ornatus* group. Measurements are given in mm: first
3 row – Min-Max values. in brackets – avarage ± Standard deviation.

Species	Number of specimens	Stridulatory length	Number of stridulatory teeth
P. affinis	9	3.68-4.46 (4.08)	122-169 (146)
P. affinis affinis	24	3.84-4.46 (4.17±0.19)	119-151 (138±12)
P. affinis hajlensis	7	4.08-4.46 (4.38±0.14)	133-153 (149±7)
P. affinis komareki	5	5.34-5.88 (5.64±0.25)	158-195 (181±15)
P. affinis serbicus	12	3.84-4.37 (4.14±0.21)	136-156 (144±6)
P. hoelzeli	8	4.14-5.34 (4.85±0.42)	125-150 (141±8)
P. jablanicensis	3	2.96-3.04 (3.01±0.05)	121-135 (128±7)
P. nobilis	15	2.78-3.98 (3.28±0.33)	81-111 (97±9)
P. nonveilleri	10	3.74-4.32 (3.97±0.18)	104-119 (111±5)
P. obesus	12	3.37-4.6 (4.28±0.31)	80-110 (92±8)
P. ornatus	10	3.74-4.6 (4.08±0.31)	105-128 (117±7)
P. pseudorantus	29	4.22-4.9 (4.66±0.16)	125-147 (139±5)
P. soulion	8	2.74-3.17 (2.99±0.13)	97-103 (99±2)
P. affinis dinaricus	1	5.38	149
P. artedentatus	1	4.8	168